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Immunization with a dicistronic plasmid expressing a truncated form of bovine herpesvirus-1 glycoprotein D and the amino-terminal subunit of glycoprotein B results in reduced gB-specific immune responses☆

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Abstract

As an approach to create a divalent DNA vaccine, a truncated secreted version of bovine herpesvirus-1 (BHV-1) glycoprotein D (tgD) and the amino-terminal subunit of glycoprotein B (gBb) were expressed from a dicistronic plasmid, designated pSLIAtgD-IRES-gBb. Intradermal immunization of mice with pSLIAtgD-IRES-gBb or a mixture of plasmids encoding tgD (pSLIAtgD) and gBb (pSLIAgBb) by needle injection or gene gun elicited strong tgD-specific immune responses. However, a significant reduction in gBb-specific immune responses was observed upon immunization of mice with pSLIAtgD-IRES-gBb or a mixture of pSLIAtgD and pSLIAgBb in comparison to immunization with pSLIAgBb alone. This reduction in gBb-specific immune responses induced by pSLIAtgD-IRES-gBb was due to production of low amounts of gBb from pSLIAtgD-IRES-gBb, inefficient processing and transport of gBb, and possibly competition for antigen-presenting cells by tgD and gBb. These results indicate that, although divalent plasmids may be used to express different antigens, the efficacy of vaccination with such plasmids may be influenced by the plasmid design and the characteristics of the expressed antigens.

Keywords: DNA vaccine; Bovine herpesvirus-1; Glycoprotein B; Glycoprotein D; Dicistronic plasmid

Introduction

DNA vaccines consist of antigen-expressing bacterial plasmids capable of inducing humoral and cellular immune responses. The kinetics, duration, and protective ability of the immune responses induced by DNA vaccines depend on several factors such as the antigen used and species studied (Donnelly et al., 1997; van Drunen Littel-van den Hurk et al., 2000). DNA vaccines have been shown to induce long-lasting protective immune responses in mice (Donnelly et al., 1997), which have been the most frequently used species to evaluate the immunogenicity and efficacy of DNA vaccines in vivo.

Bovine herpes virus-1 (BHV-1) is an economically important pathogen, which causes severe respiratory and genital infections in cattle and predisposes the animals to lethal secondary bacterial infections (Tikoo et al., 1995). Modified live viral vaccines and subunit vaccines consisting of BHV-1 surface glycoproteins such as glycoproteins B (gB) and D (gD) have been promising in protecting cattle against BHV-1 (van Drunen Littel-van den Hurk et al., 1993; Gao et al., 1994). However, although these vaccines elicit high levels of protection, they are expensive and difficult to manufacture. In comparison, DNA vaccines are relatively cost-effective and easier to design, manufacture, store, and transport. They also are safer than live viral vaccines, especially for infectious agents known to establish latency, such as herpesviruses. Finally, DNA vaccines generally induce balanced immune responses and the immune responses induced by DNA vaccines usually last longer than the responses elicited by subunit vaccines. Unfortunately,

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the efficacy of DNA vaccines seen in mice has not been observed in large animals (van Drunen Littel-van den Hurk et al., 2000). Several strategies have been developed to enhance DNA vaccine efficacy against BHV-1 in cattle. These include the use of various promoters, alternate routes of delivery (van Drunen Littel-van den Hurk et al., 1998), different antigen forms (Braun et al., 1997; van Drunen Littel-van den Hurk et al., 1998), and prime-boost regimens (Loehr et al., 2001). The results obtained with these approaches indicate that DNA vaccines may offer similar levels of protection from BHV-1 challenge as killed or modified live viral vaccines, but in contrast to subunit vaccines (van Drunen Littel-van den Hurk et al., 1993; Gao et al., 1994), they are not completely effective. Therefore, further improvements are desirable, which emphasizes the need for the design of novel vaccine vectors.

Multivalent plasmids, expressing several antigens, are being increasingly used in gene therapy and vaccination (Wild et al., 1998; Kwissa et al., 2000; Pancholi et al., 2000). Expression of different proteins may be directed by two promoters (Kwissa et al., 2000) or a single promoter with two cistrons (Wild et al., 1998). The aim of this work was to construct a dicistronic plasmid expressing two glycoproteins of BHV-1, a truncated secreted form of gD (tgD) and the amino-terminal subunit of gB (gBb), and to test the ability of the plasmid-expressed proteins to induce humoral and cellular immune responses. Truncated gD was chosen since plasmid encoding tgD induces stronger immune responses than plasmid expressing gD (van Drunen Littel-van den Hurk et al., 1998). BHV-1 gB exists as a covalently linked heterodimeric complex that can be cleaved into extracellular amino-terminal gBb (aa 1–505; 74 kDa) and carboxy-terminal gBc (aa 506–763; 55 kDa), which has a transmembrane anchor (aa 759–828). The amino-terminal subunit of gB was chosen, since at least one heparin-binding domain and several T cell and B cell epitopes have been identified in this region for mice (Fitzpatrick et al., 1990) and cattle (Gao et al., 1999). If successful, the immune responses induced to tgD and gBb should cover a broader range of protective epitopes and therefore be more effective than the responses to tgD or gBb alone. However, gBb-specific immune responses were significantly affected in mice immunized with the dicistronic plasmid or a mixture of monocistronic plasmids in a single injection. This reduction was not apparent when plasmids expressing tgD and gBb were administered in different sites.

Results

Truncated gD and gBb expressed by pSLIAtgD-IRES-gBb in transfected Cos-7 cell supernatants are recognized by gD- and gB-specific monoclonal antibodies

To determine if pSLIAtgD-IRES-gBb expresses tgD and gBb as individual proteins in eukaryotic cells, Cos-7 cells were transfected with pSLIAtgD-IRES-gBb (Fig. 1), pSLIAtgD, p3KHSPgBb, or pSLIA0. Proteins in the supernatants from the transfected cells were precipitated with a cocktail of gD-specific (Hughes et al., 1988) or gB-specific (van Drunen Littel-van den Hurk et al., 1984, 1985) monoclonal antibodies. Supernatants from untransfected (Fig. 2a, lanes 1 and 2) or pSLIA0 transfected (Fig. 2a, lanes 3 and 4) cells showed no reaction with gD-specific or gB-specific antibodies. Proteins with apparent molecular weights of 74 kDa (gBb) and 61 kDa (tgD) were precipitated from supernatants of pSLIAtgD-IRES-gBb transfected cells (Fig. 2a, lanes 5 and 6), suggesting that pSLIAtgD-IRES-gBb expresses both gBb and tgD. As expected, tgD and gBb were precipitated from supernatants of pSLIAtgD (Fig. 2a, lane 8) and p3KHSPgBb (Fig. 2a, lane 9) transfected cells, respectively. To further compare the conformation of tgD and gBb expressed by the dicistronic plasmid with that of the monocistronic counterparts, both conformation-dependent and -independent monoclonal antibodies (Clones 9D6, 136, 3E7, 10C2, 3C1, 2C8, 4C1 and 3D9S) (Hughes et al., 1988) were used to precipitate proteins produced by pSLIAtgD-IRES-gBb and pSLIAtgD. Similarly, pSLIAtgD-IRES-gBb and p3KHSPgBb transfected Cos-7 cell supernatants were precipitated with seven different gB-specific monoclonal antibodies (Clones 3F3, 1E11, 1F8, 3C7, 3G11, 5G11, 6G11) (van Drunen Littel-van den Hurk et al., 1984, 1985). All gD-specific monoclonal antibodies reacted with the 61-kDa protein in supernatants of both pSLIAtgD and pSLIAtgD-IRES-gBb transfected cells (Fig. 2b), while all gB-specific monoclonal antibodies recognized the 74-kDa protein in supernatants of both p3KHSPgBb and pSLIAtgD-IRES-gBb transfected cells (Fig. 2c). However, the reaction of the gB-specific monoclonal antibodies with gBb expressed by the dicistronic plasmid appeared to be weaker than the recognition of gBb produced by the monocistronic

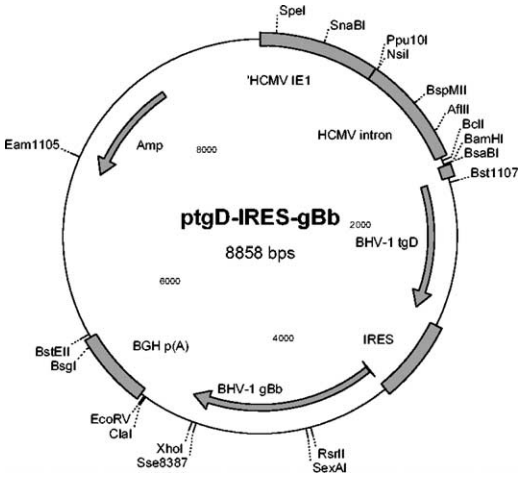

Fig. 1. pSLIAtgD-IRES-gBb. The *Pvu*II-*Eco*RV fragment containing gBb downstream of EMCV IRES was inserted behind the coding sequences of tgD in the expression vector, pSLIA0, containing the HCMV IE promoter and BGH poly (A) tail.

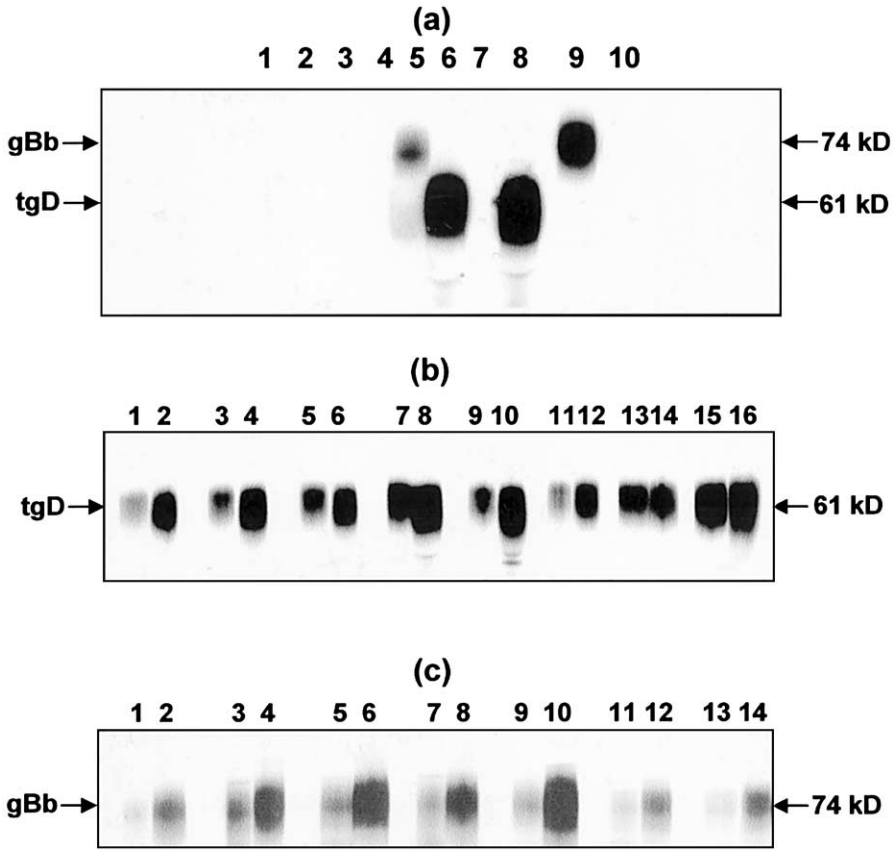

Fig. 2. In vitro expression of tgD and gBb in pSLIAtgD-IRES-gBb transfected cells. (a) Cos-7 cells were left untransfected (lanes 1, 2) or transiently transfected with pSLIA0 (lanes 3, 4), pSLIAtgD-IRES-gBb (lanes 5, 6), pSLIAtgD (lanes 7, 8), or p3KHSPgBb (lanes 9, 10). Radiolabeled proteins in culture supernatants were precipitated with a cocktail of gB-specific monoclonal antibodies (lanes 1, 3, 5, 7, 9) or gD-specific monoclonal antibodies (lanes 2, 4, 6, 8, 10). (b) Proteins were precipitated from supernatants of pSLIAtgD-IRES-gBb (lanes 1, 3, 5, 7, 9, 11, 13, 15) or pSLIAtgD (lanes 2, 4, 6, 8, 10, 12, 14, 16) transfected Cos-7 cells by gD-specific monoclonal antibody 9D6 (lanes 1, 2), 136 (lanes 3, 4), 3E7 (lanes 5, 6), 10C2 (lanes 7, 8), 3C1 (lanes 9, 10), 2C8 (lanes 11, 12), 4C1 (lanes 13, 14), or 3D9S (lanes 15, 16). (c) Proteins were precipitated from supernatants of pSLIAtgD-IRES-gBb (lanes 1, 3, 5, 7, 9, 11, 13) or p3KHSPgBb (lanes 2, 4, 6, 8, 10, 12) transfected Cos-7 cells by gB-specific monoclonal antibody 3F3 (lanes 1, 2), 1E11 (lanes 3, 4), 1F8 (lanes 5, 6), 3C7 (lanes 7, 8), 3G11 (lanes 9, 10), 5G11 (lanes 11, 12), or 6G11 (lanes 13, 14). All proteins were separated by SDS–PAGE on an 8.5% gel under reducing conditions.

plasmid. This may be due to the expression of lower amounts of gBb in pSLIAtgD-IRES-gBb transfected cells and/or overall lower reactivity of gBb as observed earlier with monoclonal antibodies 5G11 and 6G11 (Li et al., 1996b). There also appeared to be a slightly weaker recognition of gD epitopes by monoclonal antibodies 9D6, 136, 3E7, 3C1, and 2C8 (lanes 1, 3, 5, 9, 11), which may be due to conformational changes or lower expression of tgD from the dicistronic plasmid. These results suggest that although tgD and gBb are expressed from pSLIAtgD-IRES-gBb, lower amounts might be produced from the dicistronic plasmid or the expressed proteins might have some conformational changes.

Intradermal immunization of mice with pSLIAtgD-IRES-gBb induces significantly lower gBb-specific immune responses compared to immunization with pSLIAgBb

Once we demonstrated that each of our constructs was expressing the desired proteins, we investigated the ability of pSLIAtgD-IRES-gBb to elicit tgD-specific and gBb-specific immune responses in mice. The ability of pSLIAtgD to induce antibody responses in mice has been previously investigated (Braun et al., 1998). However, although gBb and gB have been shown to induce similar antibody responses (Li et al., 1996b), the immunogenicity of plasmid-expressed gBb has not been tested. Our in vitro studies were performed with p3KHSPgBb, since gBb produced by this plasmid has been previously characterized (Li et al., 1996b). However, for the purpose of immunization, we needed to control for the effect of promoter strength and plasmid backbone sequences; therefore, pSLIAgBb was constructed and tested in Cos-7 cells. The panel of gB-specific monoclonal antibodies described above identified a 74-kDa protein in supernatants of pSLIAgBb transfected cells (data not shown), confirming the expression of gBb. Subsequently, a dose titration of pSLIAgBb and pSLIAtgD-IRES-gBb was performed. C57B1/6 mice were immunized three times by intradermal injection with 2, 6, or 18 μg of either pSLI-

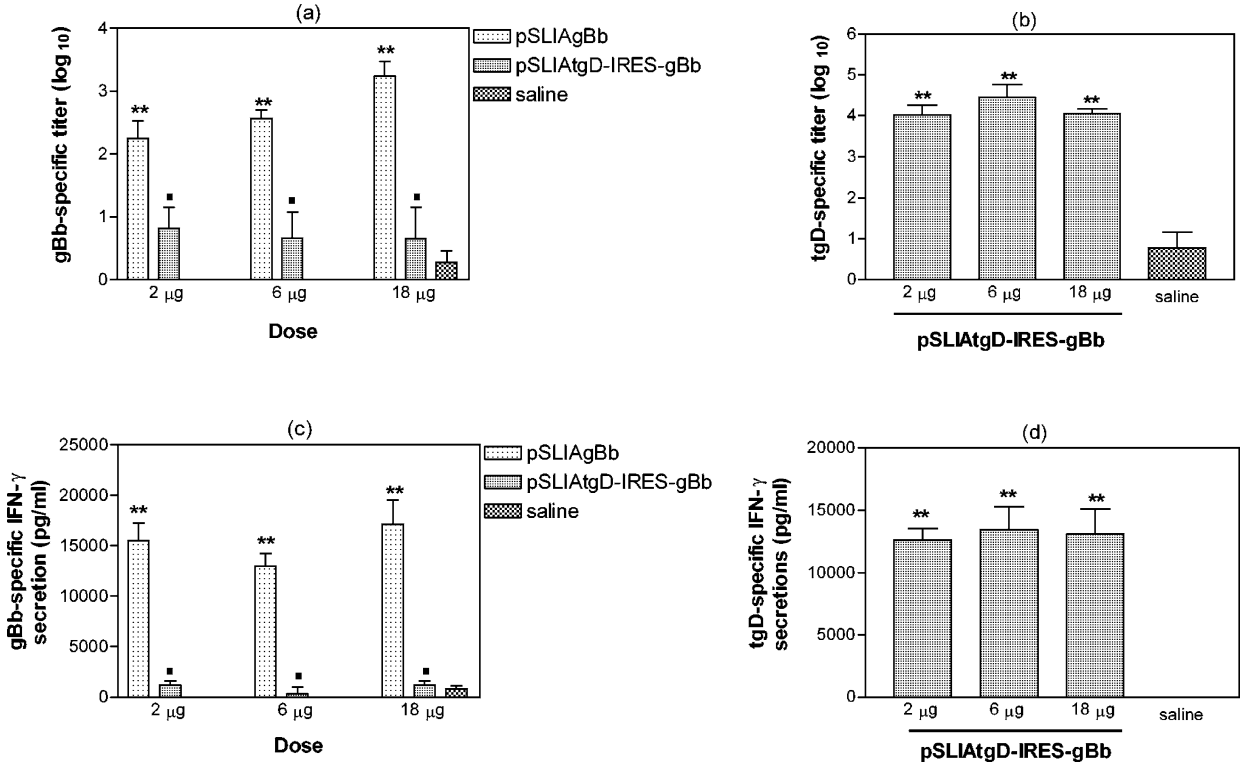

Fig. 3. Immune responses induced by intradermal injection with pSLIAgBb or pSLIAtgD-IRES-gBb. C57BL/6 mice were injected intradermally with 2, 6, or 18 μg of pSLIAgBb ($n = 5$) or pSLIAtgD-IRES-gBb ($n = 5$). All responses were measured 1 week posttertiary immunization. (a) gBb-specific and (b) tgD-specific geometric mean serum ELISA titers + SEM of pSLIAgBb and pSLIAtgD-IRES-gBb immunized mice; (c) gBb-specific and (d) tgD-specific geometric mean IFN-γ secretion + SEM of pSLIAgBb and pSLIAtgD-IRES-gBb immunized mice. ELISA titers were calculated as the reciprocal serum dilution at which the OD reading was 2× standard deviation of a standard negative serum. ** Significance of differences from the saline injected group ($P < 0.01$). ■, Significance of differences between pSLIAgBb and pSLIAtgD-IRES-gBb immunized groups ($P < 0.05$).

AgBb or pSLIAtgD-IRES-gBb. Since we anticipated that the small difference in size between the monocistronic (7139 kb) and dicistronic gBb-expressing plasmids (8858 kb) would not influence the transfection efficiency in vivo to a greater extent than the natural variation in transfection levels after intradermal injection, microgram amounts were chosen for this experiment. After the third immunization, there were significantly higher gBb-specific antibody titers (Fig. 3a) and levels of IFN-γ secretion (Fig. 3c) in mice immunized with pSLIAgBb than in mice injected with saline ($P < 0.01$), regardless of the dose of plasmid used for immunization. However, the gBb-specific antibody and cellular immune responses in mice immunized with any dose of pSLIAtgD-IRES-gBb were not different from the responses of the saline injected mice, and significantly lower than the responses of the pSLIAgBb immunized mice ($P < 0.05$) (Figs. 3a and c). In contrast, pSLIAtgD-IRES-gBb immunized mice showed significantly higher tgD-specific antibody titers ($P < 0.01$; Fig. 3b) and levels of IFN-γ secretion ($P < 0.01$; Fig. 3d) when compared to mice injected with saline. There were no detectable levels of IL-5 in any of the groups (data not shown). These results were confirmed in a second experiment. The reduction of the gBb-specific immune responses in pSLIAtgD-IRES-gBb immunized mice may be due to factors such as inefficient translation of gBb from the IRES (Mizuguchi et al., 2000), inefficient processing and transport of gBb, or antigenic competition between tgD and gBb.

Exhaustive immunoprecipitation with a gB-specific monoclonal antibody cocktail demonstrates reduced production of gBb by pSLIAtgD-IRES-gBb

In our previous experiments, there appeared to be reduced expression of gBb in pSLIAtgD-IRES-gBb transfected cell supernatants, as well as lower gBb-specific immune responses in mice immunized with pSLIAtgD-IRES-gBb. To confirm that the amount of gBb expressed by the dicistronic plasmid was indeed lower, gBb produced in supernatants of cells transfected with pSLIAtgD-IRES-gBb or pSLIAgBb was exhaustively precipitated at 24 h post-transfection. In contrast to supernatants from pSLIAgBb transfected cells, supernatants from pSLIAtgD-IRES-gBb transfected cells did not contain detectable levels of gBb after the third immunoprecipitation (Fig. 4, lanes 5 and 6). These results show that indeed the amount of gBb expressed by the dicistronic plasmid is lower than that expressed by pSLIAgBb, which may have contributed to the lower gBb-specific immune responses induced by immunization with pSLIAtgD-IRES-gBb (Fig. 3a).

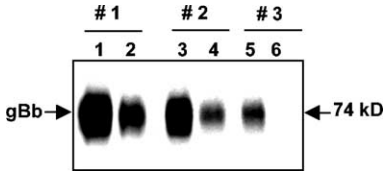

Fig. 4. Exhaustive immunoprecipitation of gBb from supernatants of pSLIAgBb and pSLIAtgD-IRES-gBb transfected cells. Radiolabeled proteins in culture supernatants of pSLIAgBb (lanes 1, 3, 5) and pSLIAtgD-IRES-gBb (lanes 2, 4, 6) transfected Cos-7 cells were precipitated three times (1, 2, and 3) with gB-specific monoclonal antibody cocktail. Proteins were separated by SDS–PAGE on an 8.5% gel under reducing conditions.

Pulse-chase analysis of pSLIAtgD-IRES-gBb shows marked reduction in the synthesis of both precursor and mature forms of gBb

Since it was evident that lower amounts of gBb were produced in pSLIAtgD-IRES-gBb transfected cells, we subsequently investigated whether this was due to reduced protein synthesis or inefficient processing and transport. To analyze the production and processing of tgD and gBb, a pulse-chase experiment was performed. Cos-7 cells were transfected with pSLIAgBb (Figs. 5a and b, lanes 1–6), pSLIAtgD (Figs. 5c and d, lanes 1–6), or pSLIAtgD-IRES-gBb (Fig. 5, lanes 7–12), and cell lysates and supernatants of the transfected cells were analyzed at various time points using gB- and gD-specific monoclonal antibody cocktails. The overall processing of gBb and tgD was similar in pSLIAtgD-IRES-gBb transfected cells compared to pSLIAgBb and pSLIAtgD transfected cells. However, there were differences in the amounts of the precursor and mature forms of gBb synthesized in cells transfected with the monocistronic and dicistronic plasmids. As shown in Fig. 5a, high amounts of gBb precursor were present at time 0 in pSLIAgBb transfected cells (lanes 1–6) in contrast to pSLIAtgD-IRES-gBb transfected cells (lanes 7–12), where increased amounts of gBb precursor were not detected until 120 min after the pulse. Fully processed gBb was first detected at about 60 min in both pSLIAgBb (Fig. 5b, lanes 1–6) and pSLIAtgD-IRES-gBb (Fig 5b, lanes 7–12) transfected cell-culture supernatants, but the amounts produced by pSLIAtgD-IRES-gBb were very low. In the cell lysates of both pSLIAtgD (Fig. 5c, lanes 1–6) and pSLIAtgD-IRES-gBb (Fig. 5c, lanes 7–12) transfected cells there was a steady conversion of tgD precursor to the mature protein from 15 min of labeling. Mature tgD was detected from 30 min onward in both pSLIAtgD and pSLIAtgD-IRES-gBb transfected cell supernatants (Fig. 5d). These results suggest that the lower amount of gBb detected in supernatants from pSLIAtgD-IRES-gBb transfected cells is due to reduced synthesis of gBb precursor, as well as less efficient processing, transport, and secretion of gBb. Interestingly, there appeared to be an accumulation of mature tgD in cells transfected with pSLIAtgD or pSLIAtgD-IRES-gBb, whereas all of the mature gBb present in cells transfected with pSLIAtgD-IRES-gBb or pSLIAgBb was secreted. Therefore, it may be possible that the presence of large amounts of mature tgD interferes with the synthesis and processing of gBb, leading to lower amounts of mature gBb in the supernatants of transfected cells.

Glycoprotein Bb-specific antibody responses are significantly reduced in mice immunized intradermally with pSLIAtgD-IRES-gBb or a mixture of pSLIAtgD and pSLIAgBb

It is very likely that the low amount of fully processed gBb expressed by pSLIAtgD-IRES-gBb contributed to the reduced gBb-specific immune responses induced by the dicistronic plasmid. However, other factors may also be involved, such as coexpression of antigen in the same cell or site. To test this possibility, mice were immunized with pSLIAgBb, pSLIAtgD, pSLIAtgD-IRES-gBb, or a mixture of pSLIAtgD and pSLIAgBb. The 6 μg dose was chosen for this experiment, since immunization with 2 μg of pSLIAgBb showed a greater variation among mice in gBb-specific antibody responses, whereas the 18 μg dose could mask any effects due to mixing of plasmids. To equalize

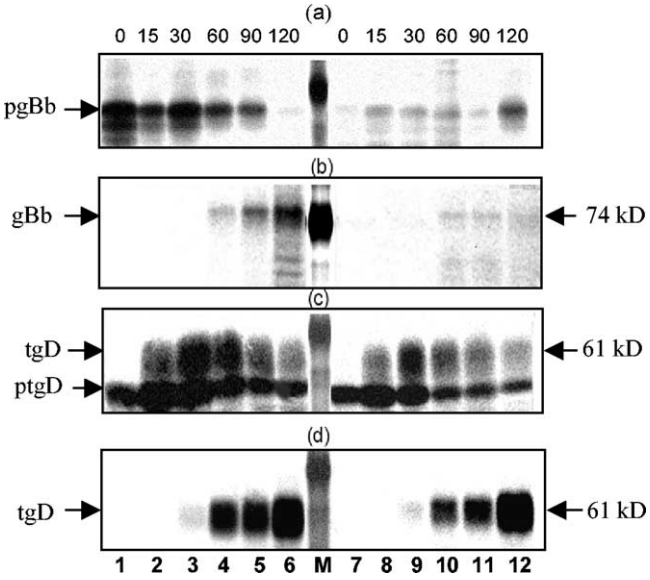

Fig. 5. Processing of tgD and gBb in pSLIAtgD-IRES-gBb, pSLIAgBb, and pSLIAtgD transfected cells. Cos-7 cells transiently transfected with pSLIAgBb, pSLIAtgD, or pSLIAtgD-IRES-gBb were labeled with ^{35}S-labeled Met/Cys for 15 min and then incubated in medium containing excess unlabeled Met/Cys for 15, 30, 60, 90, or 120 min (as indicated above the gels). (a) Cell lysates and (b) supernatants of pSLIAgBb (lanes 1–6) and pSLIAtgD-IRES-gBb (lanes 7–12) transfected cells were immunoprecipitated with gB-specific monoclonal antibody cocktail. (c) Cell lysates and (d) supernatants of pSLIAtgD (lanes 1–6) and pSLIAtgD-IRES-gBb (lanes 7–12) transfected cells were immunoprecipitated with gD-specific monoclonal antibody cocktail. Lane numbers are indicated below the gels. Precursors of gBb and tgD are indicated as pgBb and ptgD, respectively. Proteins were separated by SDS–PAGE on an 8.5% gel under reducing conditions.

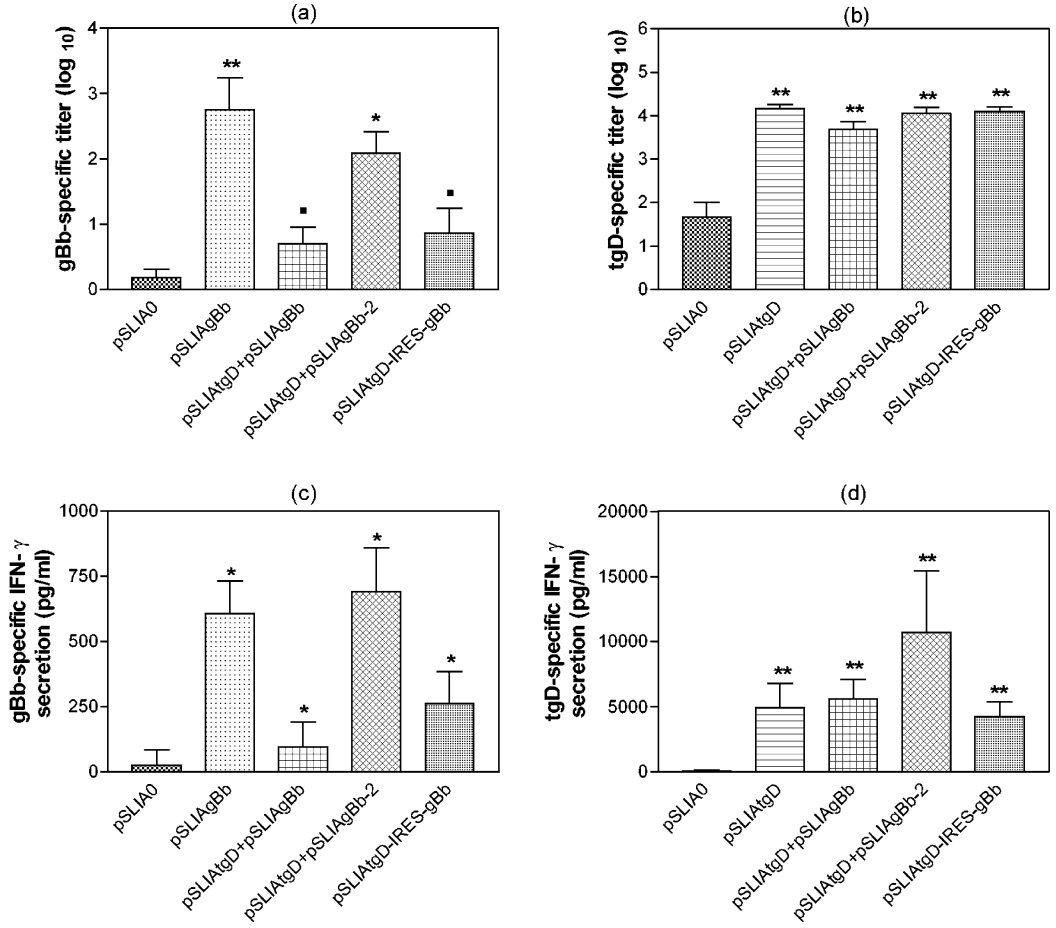

Fig. 6. Immune responses induced by intradermal injection with monocistronic or discistronic plasmids expressing tgD and gBb. C57BL/6 mice were injected intradermally with pSLIA0, pSLIAgBb, pSLIAtgD, pSLIAtgD + pSLIAgBb (one injection), pSLIAtgD + pSLIAgBb (two injections, two sites), or pSLIAtgD-IRES-gBb ($n = 7$ for all groups). Groups immunized with single plasmid also received one shot of pSLIA0 to equalize plasmid backbone in all groups. All responses were measured 2 weeks postsecondary immunization. (a) gBb- and (b) tgD-specific geometric mean serum ELISA titers + SEM; (c) gBb- and (d) tgD-specific geometric mean IFN-γ secretion + SEM. ELISA titers were calculated as the reciprocal serum dilution at which the OD reading was 2× standard deviation of a standard negative control serum. Asterisks indicate significance of differences from the pSLIA0 injected group (**$P < 0.01$; *$P < 0.05$). ■, Significance of differences between the pSLIAgBb vaccinated group and the pSLIAgBb + pSLIAtgD or pSLIAtgD-IRES-gBb immunized groups ($P < 0.05$).

plasmid backbone in all groups, pSLIA0 was added to groups that received only one plasmid. Mice vaccinated with pSLIAgBb, or pSLIAgBb and pSLIAtgD in separate sites, developed significantly higher gBb-specific antibody titers than the pSLIA0 immunized mice ($P < 0.01$ and $P < 0.05$, respectively; Fig. 6a). In contrast, there was no difference between the gBb-specific antibody responses induced by pSLIAtgD-IRES-gBb, or a mixture of pSLIAgBb and pSLIAtgD, and the responses elicited by pSLIA0. In addition, there was a significant difference ($P < 0.05$) between the gBb-specific antibody responses induced by pSLIAgBb and the responses elicited by pSLIAtgD-IRES-tgBb or the mixture of plasmids encoding gBb and tgD. All mice vaccinated with plasmid(s) expressing gBb and/or tgD, including pSLIAtgD-IRES-gBb, showed significantly higher gBb-specific IFN-γ secretion than mice vaccinated with pSLIA0 ($P < 0.05$) (Fig. 6c). Interestingly, this is in contrast to the previous experiment, where there was no significant difference in gBb-specific cellular responses between the pSLIAtgD-IRES-gBb and pSLIA0 immunized mice. This might be due to the presence of additional immunostimulatory sequences present in the plasmid backbone added in the formulation, which may have aided in enhancing cellular responses (Donnelly et al., 1997). There was no significant difference in tgD-specific antibody (Fig. 6b) and cellular immune (Fig. 6d) responses among any of the groups vaccinated with plasmid(s) encoding tgD and/or gBb. All of these groups developed significantly higher immune responses than the group injected with pSLIA0 ($P < 0.01$). These results suggest that, since there was a reduced gBb-specific antibody response when plasmids encoding tgD and gBb were delivered together, the reduction in gBb-specific responses might also be due to antigenic interference or competition for plasmid uptake.

Coimmunization of mice with pSLIAtgD and pSLIAgBb by gene gun results in decreased gBb-specific antibody responses

A consistent reduction of gBb-specific immune responses observed in mice immunized with pSLIAtgD-IRES-gBb or a mixture of pSLIAtgD and pSLIAgBb indicated a potential inhibitory effect when both proteins are expressed in the same cell or the same microenvironment. Plasmid uptake by intradermal immunization is a matter of statistical variation and this may lead to unbalanced expression of proteins, which may contribute toward lower gBb-specific antibody responses. To confirm that the reduction of gBb-specific immune responses was not due to differences in plasmid uptake, we used the gene gun to ensure delivery of both plasmids in equivalent amounts in the same cell, thereby "mimicking" the immunization with pSLIAtgD-IRES-gBb. Mice were immunized twice with gold beads coated with equal molar ratios of pSLIAgBb, pSLIAtgD, pSLIAtgD-IRES-gBb, or various combinations of pSLIAtgD and pSLIAgBb. Fig. 7 demonstrates that mice vaccinated with gBb encoding plasmid had significantly higher gBb-specific antibody titers than mice injected with pSLIA0 ($P < 0.001$). Mice immunized with pSLIAtgD and pSLIAgBb in separate sites, or with gold beads separately coated with pSLIAtgD or pSLIAgBb, developed comparable gBb-specific antibody titers to pSLIAgBb immunized mice. However, mice immunized with pSLIAtgD-IRES-gBb or gold beads coated with premixed pSLIAtgD and pSLIAgBb displayed significantly lower gBb-specific antibody titers than mice immunized with pSLIAgBb ($P < 0.05$). This may be due to a higher probability of delivering both plasmids into the same cell when gold beads are coated with premixed pSLIAtgD and pSLIAgBb than when gold beads are coated with either plasmid. These results further confirm that a reduction in gBb-specific antibody responses occurs when both tgD and gBb are produced in the same cell. All groups vaccinated with plasmid(s) encoding tgD and/or gBb had significantly higher tgD-specific antibody responses than the pSLIA0 immunized group (Fig. 7b). No significant levels of gBb- or tgD-specific IFN-γ or IL-5 secretion by splenocytes were detected in any of these groups (data not shown). Similar results were obtained when bullets were coated with a four-fold higher amount of plasmid (data not shown). These results confirm that irrespective of delivery route, a reduction in gBb-specific antibody responses occurs when tgD and gBb are expressed together in the same cell. The reduction in gBb-specific antibody responses was not as pronounced as the decrease observed after intradermal immunization. This is likely due to the fact that gene gun mediated delivery is more efficient, resulting in higher transfection efficiency. With larger numbers of plasmid producing gBb from the IRES, the relatively low amount produced per plasmid might not be as limiting to the development of an immune response as after intradermal delivery.

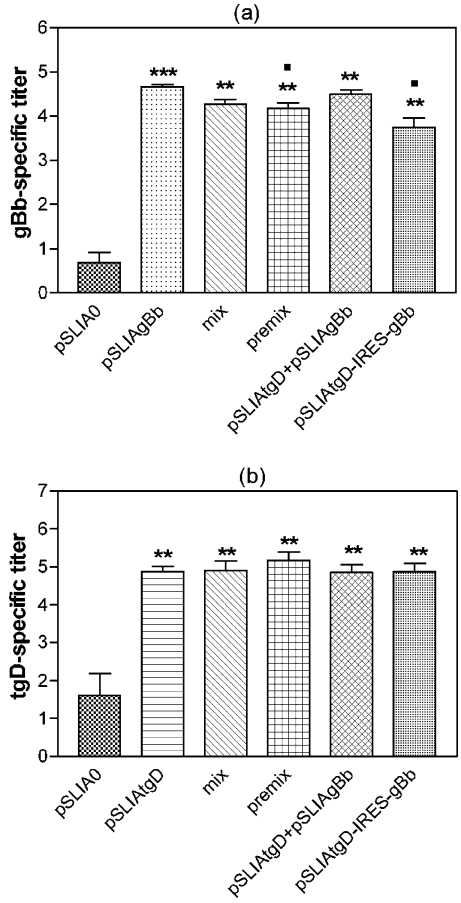

Fig. 7. Immune responses induced by gene gun mediated delivery of monocistronic or dicistronic plasmids expressing tgD and gBb. C57BL/6 mice were immunized intradermally by gene gun with (1) 6.5×10^{10} pSLIA0; (2) 6.5×10^{10} pSLIAgBb; (3) 6.5×10^{10} pSLIAtgD; (4) a combination of $\sim 3.3 \times 10^{10}$ each of pSLIAtgD and pSLIAgBb (plasmids were individually coated on gold beads, mixed, and delivered as one shot; mix); (5) 3.3×10^{10} each of pSLIAtgD and pSLIAgBb (plasmids were mixed, coated on gold beads, and delivered as one shot; premix); (6) 6.5×10^{10} pSLIAtgD + 6.5×10^{10} pSLIAgBb (delivered as two shots); or (7) 6.5×10^{10} pSLIAtgD-IRES-gBb ($n = 7$). An additional shot of 6.5×10^{10} pSLIA0 was administered to all but group 6 to equalize plasmid backbone. All responses were measured 2 weeks postsecondary immunization. (a) gBb- and (b) tgD-specific geometric mean serum ELISA titers + SEM. ELISA titers were calculated as the reciprocal serum dilution at which the OD reading was 2× standard deviation of a standard negative control serum. Asterisks indicate significance of differences from the pSLIA0 vaccinated group (***$P < 0.001$; **$p < 0.01$). ■, Significance of differences between the pSLIAgBb vaccinated group and the pSLIAgBb/pSLIAtgD premix or pSLIAtgD-IRES-gBb immunized groups ($P < 0.05$).

Discussion

Multivalent DNA vaccines are advantageous in that multiple antigens from the same or different pathogens can be expressed simultaneously. The simplest way to deliver multiple genes is administration of a mixture of monocistronic plasmids coding for the antigens of interest. However, with discovery of elements such as an internal ribosome entry site (IRES) in many viruses (Ghattas et al., 1991; Wang et al., 1993), polycistronic plasmids, which can express mul-

tiple proteins from a single mRNA, have been constructed. This approach may reduce the amount of DNA used for immunization by coexpressing different antigens from various cistrons (Kirchhoff et al., 1995). Polycistronic retroviral vectors have been used (Staal et al., 1996) in gene therapy to express cytokines such as IL-7 (Sharma et al., 1996), and granulocyte monocyte-colony stimulating factor (GM-CSF) (Guan et al., 2001) simultaneously with the gene of interest as an approach to enhance immune responses.

A dicistronic plasmid, pSLIAtgD-IRES-gBb, was constructed and tested in mice. The expression of tgD and gBb from this plasmid in Cos-7 cells was confirmed by detection with gD- and gB-specific monoclonal antibody cocktails and reactivity with individual monoclonal antibodies, indicating that both tgD and gBb retained the authentic conformation. Although there seemed to be weaker recognition by certain gD-specific monoclonal antibodies, the tgD-specific immune responses in mice immunized with pSLIAtgD-IRES-gBb were not affected. However, the overall reduced reactivity of gB-specific monoclonal antibodies with gBb expressed by the dicistronic plasmid, together with the reduced gBb-specific immune responses in mice immunized with pSLIAtgD-IRES-gBb, suggested a potential problem with the production, processing, or secretion of gBb.

Antigens encoded by a single plasmid (Wild et al., 1998; Kwissa et al., 2000) or multiple plasmids have been reported to successfully induce immune responses to all components (Grifantini et al., 1998; Musacchio et al., 2001). However, in the present study immunization of mice with dicistronic plasmid expressing tgD and gBb resulted in significant reduction in gBb-specific immune responses. Although both gBb-specific humoral and cellular immune responses were significantly lower in pSLIAtgD-IRES-gBb immunized mice, the difference in antibody responses was more pronounced. Similarly, a reduction in the gBb-specific antibody response was observed when a mixture of monocistronic plasmids encoding tgD and gBb was administered by single intradermal injection. Since effective cellular responses can be induced with low doses of antigen (O'Hagan et al., 2001), an immediate explanation for the reduced gBb-specific immune responses elicited by pSLIAtgD-IRES-gBb is the inefficient expression of gBb. Several arguments support this possibility. Exhaustive immunoprecipitation of supernatants from pSLIAtgD-IRES-gBb transfected cells with gBb-specific monoclonal antibodies demonstrated a dramatic reduction in the amount of gBb by the third time. This was confirmed by pulse-chase analysis, which showed the production of lower amounts of precursor and mature forms of gBb in cells transfected with pSLIAtgD-IRES-gBb compared to pSLIAgBb transfected cells. These results may be due to the varying translational efficiency of the IRES element (Mizuguchi et al., 2000), since the expression of gBb by the monocistronic plasmid was higher. To determine if insertion of gBb upstream of IRES enhanced expression of gBb, the positions of the tgD and gBb coding sequences were reversed and pSLIAgBb-IRES-tgD was constructed. Although the expression of gBb by pSLIAgBb and pSLIAgBb-IRES-tgD was comparable, no expression of tgD by pSLIAgBb-IRES-tgD was detected (data not shown).

Another possible explanation for the reduction of gBb expression in pSLIAtgD-IRES-gBb transfected cells may be the large accumulation of mature tgD in these cells. Although the production of high amounts of tgD may be due to the efficiency of transcription from the HCMV promoter, similar accumulation of gBb from the same promoter was not observed. Therefore, the accumulation of mature tgD in the cells may be related to the characteristic of gD that mediates resistance to further BHV-1 infection, promoting virus spread to tissues and enhancing virus infection (Chase et al., 1993; Campadelli-Fiume et al., 2000). The presence of large amounts of tgD in cells transfected in vitro or in vivo may also be expected to interfere with the processing and presentation of other proteins expressed in the same cell by saturating the glycosylation and transport machineries responsible for exporting proteins from the endoplasmic reticulum to the Golgi apparatus.

Alternatively, the characteristics of the signal peptides may play a role in determining the preference of the glycoproteins that enter the secretory pathway. The hydrophobicity of signal peptides has been shown to correlate with the ability of precursors to accomplish different stages of the secretion process (Kumamoto and Beckwith, 1985; Kumamoto, 1991). From hydophobicity analyses using the Kyte–Doolittle method (Kyte and Doolittle, 1982) with a window size of 7 aa, the signal sequence of gD (17 aa) appeared to be more hydrophobic compared to that of gB (49 aa) (Ros and Belak, 2002), possibly providing tgD with an advantage over gBb. In support of this hypothesis, Rusch et al. (1994) have shown that mutant proteins with highly hydrophobic signal peptides interfere with the secretion of wild-type proteins, suggesting a possible competition with other preproteins for one or more components of the secretory pathway. Chen et al. (1996) further showed that when two functional signal peptides are arranged in tandem, there is a strong preference to utilize the most hydrophobic one. Although these studies have been demonstrated in bacteria, the structure of signal sequences and the functioning of the secretory machinery are generally well conserved from prokaryotes to eukaryotes (Galliciotti et al., 2001). Therefore, this may also explain the presence of lower amounts of gBb in the supernatants of pSLIAtgD-IRES-gBb. An important implication of these speculations is that, when tgD and gBb are produced together in the same cell, the factors that favor the rapid expression and secretion of tgD may also lead to rapid processing followed by binding of tgD peptides to major histocompatibility complex (MHC) molecules for faster presentation and induction of an immune response.

The phenomenon of antigenic competition, in which immune responses to one determinant are inhibited by simultaneous exposure to antigens on the same or different molecules, has been known for a long time (Liacopoulos and

Ben-Efraim, 1975). Antigenic competition may occur at the level of antigen presentation. It has been suggested that competition between T cells only occurs if they are responding to different peptides presented on the same antigen-presenting cell (APC) (Kedl et al., 2000). In our experiments, immunization of mice with pSLIAtgD-IRES-gBb, or a mixture of pSLIAtgD and pSLIAgBb in a single formulation as an injection or on gold beads, might result in presentation of both tgD and gBb peptides by the same APC, causing competition between T cells. Alternatively, since animals immunized with a mixture of pSLIAtgD and pSLIAgBb or with pSLIAtgD-IRES-gBb displayed significant tgD-specific T cell responses, tgD-specific T cells could have provided help to gBb-specific B cells. However, this was not observed, so there might have been competition between tgD- and gBb-specific B cells for the T cell help, which may be another reason for the reduced gBb-specific B cell response in animals immunized with the dicistronic plasmid. In addition, APCs play a crucial role in narrowing the choice of determinants on the protein to which a response will be mounted and the nature of that response (Schneider and Sercarz, 1997). Therefore, it may also be possible that tgD possesses more immunodominant epitopes than gBb, which could initiate stronger tgD-specific immune responses that may be specific for the species studied. In support of this contention, O'Hagan et al. (2001) observed hyporesponsiveness to HIV env protein upon immunization with a combination of naked DNA encoding HIV gag and env only in the guinea pig model, and not in mice or rabbits.

In general, antibody responses have been dependent on plasmid dose used for immunization (Donnelly et al., 1997), but in some studies, this dependence seems to be lost with time as the antibody titers become equivalent irrespective of the dose (Rhodes et al., 1994) or number of injections (Deck et al., 1997). In our studies, gBb-specific antibody titers could not be enhanced in pSLIAtgD-IRES-gBb immunized animals even with doses as high as 18 μg by intradermal injection or 2 μg by gene gun. This may be explained by the fact that at the higher doses of pSLIAtgD-IRES-gBb more tgD is produced, which may result in increased competition of tgD with gBb.

Collectively, the reduction in gBb-specific immune responses in mice immunized with pSLIAtgD-IRES-gBb may be ascribed to a combination of factors, including low-translational efficiency of gBb from IRES, competition by tgD with processing, secretion and transport of gBb, and interference with the induction of gBb-specific immune responses. Plasmid-expressed gD has also been shown to have inhibitory effects in other plasmid formulations (Braun et al., 1998). However, gD still remains one of the most crucial antigens for induction of protection, so it should not be excluded from vaccines against BHV-1. Therefore, to avoid reduction in immune responses to any component of a divalent or a multivalent DNA vaccine encoding BHV-1 gD and other immunogens, it may be advantageous to immunize in multiple sites, as this may provide separate microenvironments for each antigen to induce an immune response. Alternatively, the construction of DNA vaccines encoding chimeric fusion proteins may offer a promising strategy to overcome these difficulties.

In summary, the construction of dicistronic plasmids may represent a promising approach to coexpress several gene products that may enhance the efficacy of DNA vaccines (Wild et al., 1998; Pancholi et al., 2000). Wild et al. (1998) have shown successful induction of immune responses to core and surface antigens of hepatitis virus encoded from a dicistronic plasmid. However, the results from our study suggest that the ability of each of two antigens to induce the desired immune responses in the same microenvironment depends on their individual physical properties, which may influence the intracellular synthesis and processing, and consequently the induction of immunity.

Materials and methods

All restriction enzymes, DNA-modifying enzymes, molecular weight markers, and plasmids were purchased from Pharmacia Biotech (Baie d'Urfé, QC) and New England Biolabs Ltd. (Mississauga, ON). All cell-culture incubations were performed at 37°C in 5% CO_2, unless otherwise specified.

Construction of plasmids

pSLIAtgD (6542 kb) was created by inserting the coding sequence for tgD of BHV-1 into the pSLIA0 vector (Braun et al., 1998). To construct pSLIAtgD-IRES-gBb (Fig. 1; 8858 kb), the pCITE vector bearing the sequence of equine cytomegalovirus (ECMV) IRES (Invitrogen, Burlington, ON) was used. pIRES-gBb plasmid was first constructed by inserting the *Nco*I-*Eco*RV fragment from p3KHSPgBb (8864 kb) (Li et al., 1996a) bearing the coding sequence of gBb, including the signal sequence, into the *Nco*I-*Eco*RV site on the pCITE vector to create pCITE-gBb. The *Pvu*II-*Eco*RV fragment containing IRES-gBb was inserted into the *Eco*RV site on pSLIAtgD. pSLIAgBb (7139 kb) was constructed by ligation of a Klenow-digested *Nco*I-*Hinc*II fragment of pIRES-gBb, bearing the coding sequence of gBb, into the Klenow-digested *Xba*I site of pSLIA0.

Plasmids were grown in *Escherichia coli DH5α* and purified by anion exchange resin (Qiagen, Mississagua, ON) followed by Triton X-114 (Sigma-Aldrich, Oakville, ON) extraction (Cotten et al., 1994) to reduce endotoxin levels to $<$10 EU ($<$10 pg/ml of DNA). The concentration was assessed spectrophotometrically, and constructs were confirmed by restriction enzyme digestion followed by agarose gel electrophoresis.

Transfection

Cos-7 cells were seeded at a concentration of 2.5×10^5 cells/well in six-well plates (Corning, Corning, NY) in DMEM (Canadian Life Technologies, Burlington, ON) with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and cultured overnight. They were then transfected with pSLIAtgD, pSLIAtgD-IRES-gBb, p3KHSPgBb, or pSLIAgBb using Lipofectamine Plus Reagent (Canadian Life Technologies) according to the manufacturer's instructions. After 5 h incubation with transfection mixture, DMEM containing 10% FBS was added to the transfected Cos-7 cells.

Radioimmunoprecipitation

At 24 h posttransfection, the culture medium was removed from the transfected Cos-7 cells. Fresh methionine and cysteine (Met/Cys)-free MEM (Sigma-Aldrich) was added to the transfected cells and the cells were incubated for 2 h. Subsequently, 50 μCi of ^{35}S-labeled Met/Cys (Mandel Scientific Co., Guelph, ON) was added and the cells were incubated overnight. The culture supernatants were collected and incubated with a 1:100 dilution of mouse ascites containing gB-specific monoclonal antibodies (Clones 3F3, 1E11, 1F8, 3C7, 3G11, 5G11, 6G11) (van Drunen Littel-van den Hurk et al., 1984, 1985) or gD-specific monoclonal antibodies (Clones 9D6, 136, 3E7, 10C2, 3C1, 2C8, 4C1, 3D9S) (Hughes et al., 1988) on ice for 4 h. Protein A-Sepharose beads (Amersham Pharmacia) were coated with rabbit anti-mouse IgG (Cappel, Aurora, OH) and subsequently incubated overnight at 4°C with the protein-bound gB- or gD-specific monoclonal antibodies. For exhaustive immunoprecipitations, the beads were centrifuged and supernatants were immunoprecipitated two more times. The beads were then washed and resuspended in electrophoresis sample buffer for analysis by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) on an 8.5% gel under reducing conditions.

Pulse-chase analysis

Transfection of cells with pSLIAtgD-IRES-gBb, pSLIAtgD, and pSLIAgBb was performed as described earlier. At 24 h posttransfection, cells were starved for Met/Cys in Met/Cys-free MEM (Sigma-Aldrich) for 1 h. Subsequently, cells were pulse-labeled for 15 min by addition of 200 μCi of ^{35}S-labeled Met/Cys (Mandel Scientific Co.). Supernatants were removed, and cells were washed in PBS (0.137 M NaCl, 0.003 M KCl, 0.008 M Na_2HPO_4, 0.001 M $Na_2H_2PO_4$, pH 7.4) and further incubated in MEM containing 1 mM methionine. At different time points, the cells and supernatants were collected, and samples were precipitated with gB- and gD-specific monoclonal antibody cocktails and separated by SDS–PAGE on an 8.5% gel under reducing conditions.

Immunization of mice

Four- to six-week-old C57B1/6 mice (Charles River, Kingston, ON) were randomly assigned to experimental groups and immunized either by intradermal injection on the back or by gene gun (Helios, Bio-Rad, ON) on the back and abdomen. At the end of each experiment, mice were euthanized by halothane overdose. All experiments were conducted in accordance with the guidelines provided by the Canadian Council on Animal Care.

To compare immune responses induced by various doses of pSLIAtgD-IRES-gBb, seven groups of five mice were immunized with one of the following: I: saline; II: 2 μg pSLIAgBb; III: 2 μg pSLIAtgD-IRES-gBb; IV: 6 μg pSLIAgBb; V: 6 μg pSLIAtgD-IRES-gBb; VI: 18 μg pSLIAgBb; or VII: 18 μg pSLIAtgD-IRES-gBb. Secondary and tertiary immunizations were performed in the same manner at weeks 6 and 13, respectively. At week 14, sera were collected to analyze gBb-specific antibody responses, and splenocytes were isolated to measure gBb-specific cellular responses.

To compare immune responses induced by monocistronic (pSLIAtgD and pSLIAgBb) and dicistronic (pSLIAtgD-IRES-gBb) plasmids expressing tgD and gBb, six groups of seven mice were immunized with one of the following: I: 12 μg pSLIA0; II: 6 μg pSLIAgBb + 6 μg pSLIA0; III: 6 μg pSLIAtgD + 6 μg pSLIA0; IV: 6 μg pSLIAtgD + 6 μg pSLIAgBb (one injection, one site); V: 6 μg pSLIAtgD + 6 μg pSLIAgBb (two injections, two sites); or VI: 6 μg pSLIAtgD-IRES-gBb + 6 μg pSLIA0. Secondary immunizations were performed in the same manner at week 4. At week 6, sera were collected to analyze gBb- and tgD-specific antibody responses, and splenocytes were isolated to measure antigen-specific cellular responses.

To eliminate the possibility that the reduction in gBb-specific antibody responses was due to differences in plasmid uptake, mice were immunized by gene gun. Six groups of seven mice were immunized with gold beads coated with one of the following: I: pSLIA0; II: pSLIAgBb or pSLIAtgD; III: pSLIAtgD and pSLIAgBb coated on different gold beads and delivered in a single shot; IV: pSLIAtgD and pSLIAgBb mixed prior to coating on the gold beads and delivered in a single shot; V: pSLIAtgD and pSLIAgBb delivered as two separate shots; or VI: pSLIAtgD-IRES-gBb. The conditions for plasmid delivery by gene gun were a helium pressure of 250 lb/in.^{2}, 0.25 mg of gold, and 6.5×10^{10} plasmids (ranging from 0.45 to 0.6 μg DNA) per shot. To equalize the plasmid backbone among groups, groups I, II, and VI were immunized with an extra shot containing 6.5×10^{10} pSLIA0. Secondary immunizations were performed in the same manner at week 4. At week 6, sera were collected to analyze gBb- and tgD-specific antibody responses, and splenocytes were isolated to measure antigen-specific cellular responses.

Enzyme-linked immunosorbent assay (ELISA)

Polystyrene microtiter plates (Immulon II, Dynatech Laboratories, Inc., Alexandria, VA) were coated with 0.05 μg/well of tgD (van Drunen Littel-van den Hurk et al., 1993) or tgB (Li et al., 1996b) overnight at 4°C. Plates were washed four times in PBS with 0.05% Tween 20 (PBST) prior to addition of fourfold dilutions of mouse sera prepared in PBST. After 2 h incubation at room temperature (RT), plates were washed in PBST, and affinity-purified alkaline phosphatase (AP) conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories) was added at a dilution of 1:5000 in PBST. After another hour at RT, plates were washed and reactions were visualized with 0.01 M *p*-nitrophenyl phosphate (PNPP) (Sigma-Aldrich). Absorbance was read on a model 3550 Microplate Reader (Bio-Rad Laboratories Ltd., Randolph, MA) at 405 nm, with a reference wavelength of 490 nm. The titers were calculated as the reciprocal serum dilution at which the OD reading was 2× standard deviation of a negative control serum.

Isolation of splenocytes

Isolation of splenocytes from mice has been described in detail (Lewis et al., 1999). Briefly, splenocytes were isolated by gentle homogenization of the spleens. Most red cells were removed by lysis for 1 min with ammonium chloride buffer (0.14 M NH_4Cl, 0.017 M Tris–HCl, pH 7.2). After two washes, the splenocytes were resuspended at a final concentration of 1×10^7 cells/ml in RPMI 1640 supplemented with 10% FBS (Sigma-Aldrich), 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich), 2 mM L-glutamine (Canadian Life Technologies), 100 μM nonessential amino acids (Canadian Life Technologies), 1 mM sodium pyruvate (Canadian Life Technologies), 10 mM HEPES (Canadian Life Technologies), and 5×10^{-5} M 2-β-mercaptoethanol (Sigma-Aldrich).

Cytokine ELISA

Splenocytes at a concentration of 1×10^6 cells/well were restimulated in vitro for 24 h in the presence of 3 μg/ml tgD, 1 μg/ml gD, 3 μg/ml tgB, or 1 μg/ml gB, and the culture supernatants were collected and stored at −20°C. Polystyrene microtiter plates (Immulon II, Dynatech Laboratories) were coated with 2 μg/ml of antimurine IL-5 or IFN-γ IgG (Pharmingen, San Diego, CA, USA) overnight at 4°C. Plates were washed and blocked with 1% BSA/PBS for 1 h at RT. After decanting, antigen-stimulated or untreated cell culture supernatants were added and plates were incubated for 2 h at RT. After washing, biotinylated antimurine IL-5 or IFN-γ IgG (Pharmingen) diluted to 3 μg/ml in 1% BSA/PBS was added to the appropriate wells. After 2 h incubation at RT, plates were washed in PBST, and streptavidin–alkaline phosphatase (Canadian Life Technologies) in 1% BSA/PBS was added at a dilution of 1:2500. After 1 h incubation at RT, plates were washed and reactions were visualized with 0.01 M PNPP (Sigma-Aldrich). Absorbances were read on a model 3550 Microplate Reader (Bio-Rad Laboratories) at 405 nm with a reference wavelength of 490 nm. The amounts of antigen-specific IFN-γ were calculated using doubling dilutions of IFN-γ, which were present on all plates with a range of 10 ng/ml to 5 pg/ml.

Statistical analyses

All data were analyzed with the aid of a statistical software program (GraphPad Prism version 3.0 for Windows, GraphPad Software, San Diego, CA). Prior to performance of analyses, antibody titers and IFN-γ levels were transformed by log transformation to obtain normally distributed data. One-way analysis of variance with Bonferroni's posttest was used to measure the differences between selected groups.

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